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Irreversible Thiol Oxidation in Carbonic Anhydrase III: Protection by S-Glutathiolation and Detection in Aging Rats

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Proteins with reactive sulfhydryls are central to many important metabolic reactions and also contribute to a variety of signal transduction systems. In this report, we examine the mechanisms of oxidative damage to the two reactive sulfhydryls of carbonic anhydrase III. Hydrogen peroxide (H₂O₂), peroxy radicals, or hypochlorous acid (HOCl) produced irreversibly oxidized forms, primarily cysteine sulfenic acid or cysteic acid, of carbonic anhydrase III if glutathione (GSH) was not present. When GSH was approximately equimolar to protein thiols, irreversible oxidation was prevented. H₂O₂ and peroxyl radicals both generated S-glutathiolated carbonic anhydrase III via partially oxidized protein sulfhydryl intermediates, while HOCl did not cause S-glutathiolation. Thus, oxidative damage from H₂O₂ or AAPH was prevented by protein S-glutathiolation, while a direct reaction between GSH and oxidant likely prevents HOCl-mediated protein damage. In cultured rat hepatocytes, carbonic anhydrase III was rapidly S-glutathiolated by menadione. When hepatocyte glutathione was depleted, menadione instead caused irreversible oxidation. We hypothesized that normal depletion of glutathione in aged animals might also lead to an increase in irreversible oxidation. Indeed, both total protein extracts and carbonic anhydrase III contained significantly more cysteine sulfenic acid in older rats compared to young animals. These experiments show that, in the absence of sufficient GSH, oxidation reactions lead to irreversible protein sulfhydryl damage in purified proteins, cellular systems, and whole animals.

Key words: Aging / Antioxidant / Oxidation / Reactive Oxygen Species / S-thiolation.

Introduction

Glutathione (GSH) is an essential component in the protection of cells from oxidants. It is thought to prevent oxidation of proteins by two major mechanisms. First, it may function as a direct scavenger of reactive oxygen species (Wefers and Sies, 1983; Winterbourn, 1993; Winterbourn and Metodiwela, 1994; Pichorner et al., 1995). This first function allows proteins to avoid modification during oxidative events. Second, it can form mixed disulfides with proteins, termed S-glutathiolation of proteins (Park and Thomas, 1988; Mallis et al., 2000). S-glutathiolation is a reversible oxidation of protein cysteine residues that may prevent further oxidation (Park and Thomas, 1988; Lii et al., 1994, Hamann et al., 2001). Both the oxidation of GSH to glutathione disulfide (GSSG) and the S-glutathiolation of proteins occur within seconds of addition of oxidants to cell cultures and are among the earliest measurable reactions to occur during an oxidative event (Chai et al., 1994; Li et al., 1994; Ravichandran et al., 1994; Schuppe-Koistinen et al., 1994; Dafré and Reischl, 1998). It is important to understand the relationship between direct scavenging and protein-mediated scavenging of reactive oxygen species by GSH because both mechanisms may affect the functioning of a wide range of proteins in cells during oxidative events.

Carbonic anhydrase III is a cytosolic protein that can be used as a model to study S-glutathiolation. It has two cysteine residues that are reactive to 1-chloro-2,4-dinitrobenzene (DTNB), alkylating agents, and oxidants (Engberg et al., 1985; Chai et al., 1991). It has been shown in vitro to be S-glutathiolated by hydrogen peroxide (H₂O₂), diamide, GSSG and the xanthine/xanthine oxidase H₂O₂/superoxide-generating system (Chai et al., 1991; Lii et al., 1994; Jung and Thomas, 1996). When GSH is not present to protect protein cysteines, oxidants will also cause irreversible oxidation of carbonic anhydrase III in vitro (Lii et al., 1994; Hamann et al., 2001; Thomas and Mallis, 2001). Irreversible oxidation is the formation of oxidized protein cysteine residues that are not reducible by thiol-disulfide exchange. These products may be either cysteine sulfenic acid (cysteineSO₂H) or cysteine sulfonic acid (cysteineSO₃H) (Wefers and Sies, 1983; Miller and Claiborne, 1991; Yeh et al., 1996; Becker et al., 1999; Hamann et al., 2001). H₂O₂ has been shown to produce cysteine sulfenic acid in creatine kinase and both sulfenic and sulfonic acid in carbonic anhydrase III (Hamann et al., 2001). Cysteine sulfenic and sulfonic acid are present in a wide range of purified proteins and cysteine.
sulfenic acid is found in total protein extracts of rat liver (Hamann et al., 2001), and so the formation of cysteine sulfenic or sulfonic acid may be a general mechanism for damage to proteins by oxidants.

Differences in protein cysteine oxidation (reversible and irreversible) which occur with changing GSH levels may be responsible for redox regulation of cellular processes (Storz et al., 1990; Sen and Packer, 1996; Cotgreave and Gerdes, 1998), although the mechanism for oxidant-mediated regulation of individual proteins is not well-characterized on a molecular level. Cellular glutathione can affect such diverse processes as transcrption (Storz et al., 1990; Sen and Packer, 1996), apoptosis (Cotgreave and Gerdes, 1998), damage to DNA (Park et al., 1998), cell division (Park et al., 1998), proliferation of cancer cells (Terradez et al., 1993), susceptibility to diseases (Terradez et al., 1993; Herzenberg et al., 1997; Müller and Gebel, 1998), and enzyme activity (Thomas et al., 1995b). GSH was presumed to regulate these processes either by acting as an antioxidant, preventing oxidation of critical cysteines (Winterbourn and Metodiewa, 1999), or through formation of GSSG and subsequent S-thiolation of the proteins involved (Dröge et al., 1994). Because GSSG levels in cells rarely reach levels necessary for S-glutathiolation, other mechanisms are necessary to explain S-glutathiolation in vivo. In fact, available evidence suggests that proteins are better scavengers of oxidants than low molecular weight thiols such as GSH (Mallis et al., 2001). There is no reason to suspect that glutathione is a required intermediary between oxidant and protein; instead, it most likely functions to provide a free reactive thiol where steric factors prevent formation of a protein-protein disulfide. Carbonic anhydrase III is in fact S-glutathiolated in hepatocytes without corresponding increases in GSSG levels (Chai et al., 1994). When GSH is depleted from hepatocytes using the γ-glutamylcysteine synthase inhibitor buthionine sulfoximine (BSO), the radical generator menadione causes irreversible oxidation of carbonic anhydrase III (Chai et al., 1994). Thus, at normal GSH concentrations, reduced and S-glutathiolated forms of protein should predominate after oxidation. Irreversibly oxidized proteins may predominate when GSH concentrations are low. For example, it has been proposed that tyrosine phosphatase is reversibly regulated by S-glutathiolation, and that S-glutathiolation prevents permanent inactivation of this protein by reactive oxygen species (Barrett et al., 1999a, b).

The effects of three different oxidants, H₂O₂, 2,2'-azobis(2-amidino propane)dihydrochloride (AAPH), and hypochlorous acid (HOCl) on carbonic anhydrase III are studied here. This study attempts to determine the role of GSH concentration in the protection of protein cysteine residues from irreversible oxidation. In particular it defines at what concentrations GSH reacts directly with oxidants to prevent protein oxidation, and at what concentrations it participates in S-thiolation reactions. Differences between three oxidants, H₂O₂, AAPH, and HOCl, are explored in this context. Experiments are also performed in intact hepatocytes to demonstrate the validity of the in vitro model system. Evidence shows that proteins are good scavengers of oxidants relative to GSH, and that a major role of GSH in protection of proteins is in S-glutathiolation reactions. Furthermore, irreversibly oxidized forms of carbonic anhydrase III are more abundant in normal aged rat tissue as well as in oxidant treated hepatocytes when GSH is depleted.

Results

GSH Protects Carbonic Anhydrase III from Irreversible Oxidation by H₂O₂

Purified carbonic anhydrase III is modified specifically on up to two cysteine residues following addition of 1-chloro-2,4-dinitrobenzene (DTNB), GSSG, diamide, t-butyl hydroperoxide, and xanthine/xanthine oxidase (Engberg et al., 1985; Chai et al., 1991; Lii et al., 1994; Jung and Thomas, 1996). It is modified in cells treated with diamide, menadione, and t-butyl hydroperoxide (Lii et al., 1994; Jung and Thomas, 1996).

H₂O₂ reacts readily with thiols to form sulfenic acid which reacts readily with available thiols to form sulfides (Figure 1). In the absence of available thiols, sulfenic acid will continue to react with H₂O₂ to form sulfenic acid and sulfonic acid (Miller and Claiborne, 1991; Yeh et al., 1996; Hamann et al., 2001). Carbonic anhydrase III reaction with oxidants was analyzed by isoelectric focusing (IEF). In IEF gels, addition of negative charge to the protein by either S-glutathiolation or oxidation to sulfenic and sulfonic acids, results in a more acidic protein band. Reversible modifications are defined by the loss of the acidic protein band on reduction with DTT. Irreversible modifications are resistant to DTT reduction. IAM, NEM and DTT were equally effective for preventing any oxidation of carbonic anhydrase III.

When carbonic anhydrase III was incubated with H₂O₂ for 15 minutes (Figure 2A), acidic bands with pI’s of 7.2 and 6.8 appeared. These bands were mostly irreversible to DTT reduction (indicated by the 5% change between lanes 2 and 3), suggesting the cysteines were almost entirely irreversibly oxidized. When oxidized with H₂O₂ in the presence of GSH (lanes 4 and 5), carbonic anhydrase III was modified to a greater extent and the acidic forms were mostly sensitive to DTT. A minor amount of the reactive protein cysteine (~10%) remained insensitive to

![Fig. 1 The Reaction Mechanism of H₂O₂ with Sulphydryls.](Image)
DTT. Fully S-glutathiolated carbonic anhydrase III migrates with a pI of 6.8 (lane 6). When this thiolated form of carbonic anhydrase III was treated with H₂O₂, no additional bands appeared on the gel, and all modification was completely reversible (lanes 7 and 8). These results indicated that a disulfide bond between carbonic anhydrase III and GSH prevented oxidation of the protein cysteine residues to sulfinic and sulfonic acids.

When H₂O₂-treated carbonic anhydrase III was analyzed for protein sulfinic and sulfonic acid, the amount of sulfinic acid was comparable to the amounts of irreversibly oxidized carbonic anhydrase III detected by IEF (Figure 2B). Sulfonic acid was not detected in these samples (Detection limit=2.5%).

Fig. 2  GSH Prevents Irreversible Oxidation of Carbonic Anhydrase III by H₂O₂.
(A) Carbonic anhydrase III (30 µM) or S-glutathiolated carbonic anhydrase III was incubated with 2 mM H₂O₂ for 15 min at 37 °C in 20 mM β-glycerophosphate buffer pH 7.4. Carbonic anhydrase III was treated with H₂O₂ in either the absence or presence of 0.3 mM GSH. The reactions were stopped by treating the samples with 40 mM IAM. A portion of each was reduced with 10 mM DTT for 30 min at 37 °C before addition of IAM. (B) Carbonic anhydrase III was analyzed for irreversibly oxidized cysteine by densitometry of IEF gels or protein sulfinic and sulfonic acid analysis as described in Materials and Methods. Irreversibly oxidized cysteine was found to be entirely sulfinic acid; sulfonic acid was not detected in these samples. (Detection limit=2.5%).

Fig. 3  Oxidation of Carbonic Anhydrase III with H₂O₂ and GSH.
Carbonic anhydrase III (10 µM) with the indicated concentration of GSH was incubated with 2 mM H₂O₂ at 37 °C in 20 mM sodium phosphate buffer pH 7.4. Reactions were stopped at the indicated time by incubating the reaction mixtures with 20 mM N-ethylmaleimide (NEM). Carbonic anhydrase III was reduced (right half of each gel) by incubating the reaction mixtures with 10 mM DTT for 20 minutes followed by addition of 20 mM NEM. IEF separations were performed as described in Materials and Methods. (A) Carbonic anhydrase III+H₂O₂ without addition of GSH. (B) Carbonic anhydrase III+450 µM GSH+H₂O₂. (C) Analysis of the reaction between carbonic anhydrase III, GSH and H₂O₂. Modified cysteines of carbonic anhydrase III were determined by analysis of the IEF separations in panels (A) and (B) of this Figure as well as a similar experiment with 150 µM GSH (not shown) as described in Materials and Methods.
The concentration of GSH needed to protect carbonic anhydrase III from H₂O₂-mediated damage was determined at two concentrations (10 µM and 30 µM) of carbonic anhydrase III. Figure 4A shows that reversible modification (open symbols) reached nearly maximal levels, and the protein was protected from irreversible oxidation (closed symbols) by 50 µM GSH at both protein concentrations. At GSH concentrations below 50 µM, irreversible oxidation of carbonic anhydrase III was dependent on carbonic anhydrase III concentration. H₂O₂ caused more irreversible oxidation at 5 µM and 15 µM GSH at the higher concentration of carbonic anhydrase III. Complete protection of 30 µM carbonic anhydrase III from irreversible oxidation required 50 µM GSH, while 10 µM carbonic anhydrase III was maximally protected at 15 µM GSH. Thus, protection of proteins by GSH was efficient when GSH:protein molar ratios approached one. When 5 µM GSH was present, S-glutathiolated protein accounted for 50% and 100% of the GSH available in reactions of H₂O₂ with 10 µM and 30 µM carbonic anhydrase III, respectively. Thus, when GSH is limiting, S-glutathiolation is likely to be the primary function of GSH in protection of carbonic anhydrase III from irreversible oxidation by H₂O₂. At 1.3 mM GSH, 50% of the carbonic anhydrase III was S-glutathiolated, and none was irreversibly oxidized (data not shown). Carbonic anhydrase III was modified by H₂O₂ even when GSH concentrations were 130 times greater than the protein concentration.

GSH Protects Carbonic Anhydrase III from Irreversible Oxidation by the Peroxyradical Generator AAPH

AAPH is a compound that generates alkyl peroxo radicals by thermal decomposition (Figure 5). It has been used as

![Fig. 4](image.png)

**Fig. 4** Effect of GSH Concentration on Carbonic Anhydrase III Modification by H₂O₂.
Carbonic anhydrase III (10 µM or 30 µM) with the indicated concentrations of GSH was incubated with 2 mM H₂O₂ for 10 minutes at 37 °C and in 20 mM sodium phosphate buffer at pH 7.4. Reactions were stopped, carbonic anhydrase III was reduced, and results were analyzed as in Figure 3.

![Fig. 5](image.png)

**Fig. 5** The Reaction Mechanism of AAPH.
AAPH splits homolytically at 37 °C to form 2 mol of alkyl radical per mol of AAPH (Niki, 1990), which then react quickly with molecular oxygen to form peroxy radicals.

![Fig. 6](image.png)

**Fig. 6** Oxidation of Carbonic Anhydrase III by AAPH.
(A) Carbonic anhydrase III or S-glutathiolated carbonic anhydrase III (30 µM) was incubated with 50 mM AAPH for 20 min at 37 °C in 50 mM sodium phosphate buffer pH 7.4. All other conditions were the same as described in Figure 2A, except that reactions were stopped with 20 mM NEM. (B) The amount of irreversibly oxidized cysteine found in AAPH oxidized carbonic anhydrase III was determined by densitometry of IEF gels or protein sulfenic and sulfonic acid analysis. Irreversibly oxidized cysteine was determined to be 25% sulfenic acid, 75% sulfonic acid in samples treated with AAPH alone; 37% sulfenic acid, 63% sulfonic acid in samples treated with AAPH in the presence of GSH.
a model for peroxyradical-mediated oxidation both in vitro and in vivo (Thomas et al., 1995a; Gesquière et al., 1999; Marangon et al., 1999; Wang and Joseph, 1999).

AAPH-mediated oxidation produced protein bands with the same acidic pI's as those produced by H$_2$O$_2$ (Figure 6A, lanes 2 and 3) and an additional band with a more alkaline pI. These bands were largely insensitive to DTT reduction. A protein band with a more alkaline pI could result from the radical addition of a positively charged amidinopropane group from AAPH, or by the loss of a negative charge by decarboxylation of an acidic amino acid. It is interesting to note that either S-glutathiolation (Figure 6A) or NEM pretreatment (not shown) of carbonic anhydrase III prevented formation of the positively charged band, suggesting that protein cysteine residues are involved. The modification affected less than 10% of the protein.

In the presence of GSH, AAPH produced reversible oxidation (lanes 4, 5) while irreversible oxidation was almost entirely prevented. When fully S-glutathiolated carbonic anhydrase III was treated with AAPH, no additional modification of the protein occurred (lanes 7, 8). Figure 6B compares the amount of sulfenic and sulfonic acid in AAPH-oxidized carbonic anhydrase III to the amount of oxidized protein determined by IEF. Unlike oxidation with H$_2$O$_2$, AAPH-treated carbonic anhydrase III produced a considerable amount of cysteine sulfonic acid. Seventy-five percent of the irreversibly oxidized cysteine detected from AAPH-treated carbonic anhydrase III was recovered as sulfonic acid. The presence of GSH decreased the total amount of irreversibly oxidized cysteine detected, and 63% of this was recovered as sulfonic acid. Sulfenic and sulfonic acid were not detected in untreated carbonic anhydrase III or in AAPH treated S-glutathiolated carbonic anhydrase III (data not shown).

Since the ratio of GSH to protein is a critical factor in preventing oxidative damage to proteins, the effect of GSH on AAPH oxidation was examined in detail. When GSH was varied from 0 to 150 µM (a five-fold molar excess of GSH), the amount of irreversible damage decreased to less than 5%, while the amount of reversible oxidation was nearly a mirror image of that effect (Figure 7A). Total modification of carbonic anhydrase III remained quite constant (approximately 30% of the reactive cysteines were modified). When GSH was raised to 1.3 mM, total protein modification decreased as a result of the scavenging effect of the free glutathione pool on peroxyradicals (data not shown). The amount of modification caused by AAPH in the presence of GSH could not be accounted for by the concentration of GSSG produced by direct oxidation of that molecule (not shown).

When carbonic anhydrase III concentration was varied from 30 µM to 240 µM during AAPH oxidation with 150 µM GSH, reversible modification increased in proportion to carbonic anhydrase III concentration (figure 7B). Irreversible modification was negligible in this experiment (data not shown). S-glutathiolation was about 30% at 30 µM carbonic anhydrase III and decreased only slightly to about 20% modification when carbonic anhydrase concentration was 240 µM. Since the amount of S-glutathiolated carbonic anhydrase III increased with carbonic anhydrase III concentration, it appears that carbonic anhydrase III trapped peroxyradicals very efficiently. When the concentration of carbonic anhydrase III was 240 µM, there was 40 µM of protein bound glutathione, accounting for 25% of the total GSH available. S-glutathiolated protein therefore accounts for a significant fraction of oxidized glutathione in this experiment.

**GSH Does Not Protect HOCl-Treated Carbonic Anhydrase III by S-Glutathiolation**

Although HOCl oxidizes GSH to the sulfonamide (Winterbourn and Brennan, 1997), less is known about its effect on protein thiols. The sulfenyl chloride has been proposed as a possible intermediate in the oxidation of GSH (Winterbourn and Brennan, 1997; Pullar et al., 2001). It seems likely that such an intermediate would occur with protein thiols, as well. In the presence of excess GSH, this intermediate might react to produce an S-glutathiolated protein.

When carbonic anhydrase III was oxidized with HOCl (Figure 8A, lanes 2 and 3) the protein migrated to the lower pI indicative of irreversibly oxidized carbonic anhydrase III. The extent of irreversible oxidation determined by IEF was in agreement with values determined by analysis of protein sulfenic and sulfonic acids (Figure 8B).
of which protein sulfonic acid represented 41% of the total. However, in the presence of excess GSH, about 8% of the protein reactive cysteine was reversibly modified (lanes 4 and 5), suggesting that protein S-glutathiolation is not a major mechanism occurring with HOCl. When these samples were analyzed for protein sulfinic and sulfonic acid, 20% of the reactive cysteine was irreversibly oxidized, mostly to the sulfonic acid (75% of the total irreversibly oxidized cysteine). The discrepancy between the IEF analysis and the protein sulfinic and sulfonic acid analysis could result from the formation of a charge neutral adduct, possibly a form of sulfinamide (Raftery et al., 2001), which was converted to a sulfonic acid during acid hydrolysis of the protein sample. If this hypothesis is correct, GSH may simply trap HOCl, thus delaying oxidation of a reactive intermediate to the sulfinic or sulfonic acid and allowing the putative sulfinamide to form. Since this modification apparently results in the formation of sulfonic acid in protein samples with no apparent charge differences, it might in fact be detectable by these characteristics in any protein sample under study. This concept is of interest with respect to the potential oxidative modifications that might occur in vivo (Pullar et al., 2001).

Cysteine Acts via S-Thiolation to Protect Carbonic Anhydrase III Thiol s from Oxidative Damage

GSH has been shown to be the major thiol participating in S-thiolation reactions in cells (Chai et al., 1994). This may be because of a special affinity of protein S-thiolation sites for GSH, or because of the relatively high concentration of GSH compared to other low molecular weight thiols in cells (Mallis and Thomas, 2000). We therefore studied whether cysteine would also protect carbonic anhydrase III from oxidative damage by HOCl. When carbonic anhydrase III was incubated with H₂O₂, 15% of the reactive cysteines were modified by a single negative charge (Figure 9, second bar). Cysteine prevented this modification by H₂O₂ as shown by its nearly complete inhibition of negative charge modification (third bar). In order to be able to detect the neutrally charged S-cysteylated carbonic anhydrase III, the protein was reacted with the negatively charged alkylation agent iodoacetic acid (IAA). S-cysteylation appears in this assay as an inhibition of IAA derivitization, while irreversible oxidation and IAA derivitization are indistinguishable (Thomas et al., 1995a). When carbonic anhydrase III reacts with IAA, it is modified with a negative charge on 50% of the reactive sites (fourth bar), which agrees with previously published reports of rat liver carbonic anhydrase III (Thomas et al., 1995a). If carbonic anhydrase III is incubated with H₂O₂ before IAA derivitization, there is a modest increase in negatively charged modification (fifth bar). This small increase indicates that little of the modification caused by H₂O₂ occurred on the thiol which is does not react with IAA. Only 15% of the reactive cysteines are modified with

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Fig. 8 HOCl Causes Irreversible Oxidation But Not S-Glutathiolation of Carbonic Anhydrase III.

(A) Carbonic anhydrase III or S-glutathiolated carbonic anhydrase III (30 μM) was incubated with 150 μM HOCl for 20 min at 37 °C in 50 mM sodium phosphate buffer pH 7.4. All other conditions were the same as described in Figure 6. (B) The amount of irreversibly oxidized cysteine found in HOCl oxidized carbonic anhydrase III was determined by densitometry of IEF gels or protein sulfinic and sulfonic acid analysis. Irreversibly oxidized cysteine was determined to be 59% sulfonic acid, 41% sulfonic acid in samples treated with HOCl alone; 25% sulfonic acid, 75% sulfonic acid in samples treated with HOCl in the presence of GSH. The error in the irreversible oxidation analysis by IEF is smaller than the linewidth in this illustration.

Fig. 9 Protection of Carbonic Anhydrase III from H₂O₂ Oxidation by Cysteine.

Carbonic anhydrase III (10 μM) was incubated with 2 mM H₂O₂ in the absence and presence of 150 μM cysteine at 37 °C and at pH 7.4 for 10 minutes. The reactions were stopped with either 10 mM NEM (open bars) or 10 mM IAA (crosshatched bars) as indicated. IEF was performed as described in Materials and Methods. Negative charge modification was then calculated as the% modification as described in Materials and Methods.
a negative charge when cysteine is present during the reaction of \( \text{H}_2\text{O}_2 \) with carbonic anhydrase III (sixth bar), indicating that more than 35% of the sites are S-cysteylated. Therefore cysteine also acts to protect proteins via S-thiolation.

**GSH Protects Carbonic Anhydrase III from Irreversible Oxidation in Hepatocytes**

Since relatively small changes in the molar ratio of GSH to protein sulfhydryls can have significant effects on the protection of reactive protein sulfhydryls, the protein modifications occurring in oxidant-treated rat hepatocytes were studied. Because hepatocytes contain a large amount of endogenous carbonic anhydrase III, they provide a suitable model to examine modification of that protein *in vivo*.

Cellular glutathione pools of primary hepatocytes were depleted with DEM, a substrate for cellular glutathione S-transferase, and oxidative stress was initiated with menadione, a redox cycling compound that generates reactive oxygen species. It has previously been shown that menadione promotes S-glutathiolation of carbonic anhydrase III as well as other hepatocyte proteins (Lii et al., 1994). Figure 10 describes the experimental conditions for depletion of total glutathione by DEM. After 20 minutes, 0.03 mM DEM depleted total glutathione to 80% of normal, while 2 mM DEM depleted total glutathione to 15% of normal. After a medium change, the cells were incubated with 2 mM menadione for 15 minutes and then the medium was again changed. Total glutathione remained at approximately the same level or increased slightly after DEM-containing medium was removed. Menadione had no measurable effect on total glutathione. DEM caused no increase in cell lysis as measured by lactate dehydrogenase activity found in the culture medium and menadione caused only a slight (5%) increase even two hours after its addition to cells (not shown). Thus, the initial loss in total glutathione with DEM treatment was not the result of a loss of cellular integrity.

Modified forms of carbonic anhydrase III in cells were monitored by IEF coupled with Western blot analysis (Lii et al., 1994; Thomas et al., 1995a). Figure 11A shows an analysis of carbonic anhydrase III after DEM and menadione treatment of hepatocytes. Lanes 1 and 2 are samples of pure carbonic anhydrase III which show the positions of the reduced, singly and doubly S-glutathiolated forms of the protein. A single irreversibly oxidized form of carbonic anhydrase III was not observed. Therefore, the protein modifications occurring in oxidant-treated rat hepatocytes were studied.

**Fig. 10** Effects of DEM and Menadione on Hepatocyte Glutathione. Hepatocytes were first incubated in medium B for 1 h, and DEM was added to a final concentration of 0.03 mM or 2 mM. After 20 min the medium was replaced with fresh medium B and 0.2 mM menadione was added 15 min later. After 15 min medium was replaced with fresh medium A. At the times indicated, cells were rinsed twice with cold buffered saline and lysed as described in Materials and Methods. (A) Glutathione is expressed as the mean of duplicate cultures. Duplicates varied by less than 6%.

**Fig. 11** Effects of Menadione on Hepatocyte Carbonic Anhydrase III in DEM-Treated Cultures. Rat hepatocytes were treated with DEM followed by menadione as described in Figure 10. Carbonic anhydrase III was separated by electrofocusing and detected by Western blot analysis. A portion of each sample was treated with 50 mM dithiothreitol before separation by electrofocusing. Each pair of lanes shows the carbonic anhydrase III forms in an untreated and a reduced sample (50 mM dithiothreitol) of the hepatocyte protein mixtures indicated below. Each lane contained 0.5 µg total proteins. In panel (A), lanes 1 and 2 show the reduced, partially S-glutathiolated, and fully S-glutathiolated forms of carbonic anhydrase III. Lanes 3,4 contain the protein mixture from untreated hepatocytes. Lanes 5,6 show proteins from cells treated with 2 mM DEM for 20 min. Lanes 7 and 8 are proteins from untreated cells exposed to 0.2 mM menadione for 15 min. In panel (B) hepatocytes were pretreated with either 0.03 mM DEM (lanes 1 – 4) or 2 mM DEM (lanes 5 – 8) followed by 0.2 mM menadione for 15 min. At the times indicated (0 time is the time when menadione was added to the culture) hepatocytes were washed and protein extracts were prepared.
carbonic anhydrase III resistant to reduction by DTT was found in untreated hepatocytes (lanes 3,4). Serial dilution of these samples shows that nearly one third of the protein contains a single irreversibly oxidized cysteine (data not shown). DEM (lanes 5,6) had no effect on carbonic anhydrase III, while menadione caused a reduction-sensitive oxidation on up to two cysteine residues in hepatocytes (lane 7, 8) as reported previously (Lii et al., 1994).

DEM and menadione were used in combination in the experiment shown in Figure 11B. Menadione potentiated a rapid oxidation within 15 minutes after 0.03 mM DEM treatment (lane 1) that was not completely reversible with DTT treatment (lane 2, compare to panel A, lane 8). Two hours after the addition of menadione, S-glutathiolation had disappeared (lane 3) while irreversible oxidation appears unchanged from that at the initial observation (compare lane 2 and 4). In a similar experiment where glutathione was more extensively depleted by 2 mM DEM, menadione produced less S-glutathiolation (lane 5) and considerably more irreversible oxidation (lane 6, compare to panel A, lane 8). Two hours later, there was no S-glutathiolated protein and again the irreversibly oxidized forms persist (lanes 7,8).

Irreversible Oxidation of Protein Cysteines Occur Increasingly with Age

One of the hallmarks of the aging process is a decrease in overall GSH levels (Hagen et al., 1999). Since glutathione was necessary to protect carbonic anhydrase III via S-glutathiolation in hepatocytes, it was thought that aged animals, containing less glutathione than normal, might have increased protein sulfenic and sulfonic acid. Table 1 shows that the soluble protein fraction from young rat livers (1 month old male and female) contained cysteine sulfinic acid equivalent to approximately 0.8 mol % of the total protein cysteine content. The livers of older male animals (16 months) contained approximately 1.3 mol % cysteine sulfinic acid, i.e., a 62% increase. The difference between young and old female rats was significantly different by the student’s t-test. Protein sulfonic acid was below detection limits in these samples (<0.15%).

When the carbonic anhydrase III of old rats was compared to that in young animals, increased irreversible oxidation was observed (Figure 12). Two gel-based separation methods gave similar results in this experiment. First, MalPEG (Wu et al., 2000) was used to derivatize the protein sample since it increases the overall molecular weight of a protein by 5 kDa per reactive thiol (Figure 12A). The apparent molecular weight of the MalPEG-tagged protein appears higher than expected when analyzed by SDS-PAGE. The addition of the large, uncharged polyethylene glycol group probably retards the migration of a protein through the gel since electrophoretic mobility is a function of both protein charge and size. The reagent does not react with cysteine residues that are irreversibly oxidized, and irreversibly oxidized forms of carbonic anhydrase can be separated from undamaged protein by

| Table 1 | The Effect of Aging on Rat Liver Sulfinic Acid Content. |
| Soluble liver protein extract | Cysteic acid (%) of total cysteine | Calculated sulfinic acid (B–A) |
| | A | B with NaOCl |
| Male | | |
| 1 month | N.D. | 0.80±0.14 | 0.80±0.14 |
| 16 month | N.D. | 1.11±0.23 | 1.11±0.23 |
| Female | | |
| 1 month | N.D. | 0.83±0.17 | 0.83±0.17 |
| 23 month | N.D. | 1.29±0.20 | 1.29±0.20 |

*Liver extract was prepared and analyzed for protein sulfenic and sulfonic acid as described in Materials and Methods. Protein extracts were made from 3 rats for each sex and age group. N.D. = not detected (<0.15% of total cysteine). There was a significant difference between young and old female rats (t<0.05).
SDS-PAGE Western blots. As described previously (Cabisco and Levine, 1995), the carbonic anhydrase III level was five-fold higher in young rats than in old rats (data not shown). It was necessary to normalize the samples from young and old rat for this difference in carbonic anhydrase III. In a control experiment, pure carbonic anhydrase III, added to young and old rat liver extracts, migrated as a single band on SDS-PAGE gels with a molecular size of 30 kDa, while MalPEG-treated carbonic anhydrase III was approximately 72 kDa (not shown). The left lane in Figure 12A shows a sample in which equal amounts of reduced carbonic anhydrase III were mixed with MalPEG-derivatized carbonic anhydrase III before gel separation. The two lanes on the right were obtained from MalPEG-treated protein extracts from young and old animals. The carbonic anhydrase III from older rats contained an increased amount of carbonic anhydrase III that did not react with MalPEG (two bands with lower molecular size). An analysis of these protein samples by IEF gels gave similar results (Figure 12B). The sample from old rats showed a significant increase in the amount of protein focusing with a pI of 7.2, i.e., protein with one irreversibly oxidized reactive sulfhydryl, and even a faint band with a pI of 6.8 that corresponds to protein with damage to both reactive sulfhydryls.

Gel-based methods of analysis can be quantified by densitometry, but since MalPEG-derivitization produces a significant broadening of the derivatized band it is not as amenable to such evaluation as the electrofocusing gels. Quantification of the IEF gel results showed that the carbonic anhydrase III in young rats contained 1.5% of the total cysteine as irreversibly oxidized forms while the old rats contain approximately 2.5% oxidized. Since only two of the cysteines in carbonic anhydrase III are reactive and the protein contains 5 cysteines, the observed oxidation represents 3.7% of the reactive cysteine in young animals and 6.2% in old animals. When these data are compared to the total protein cysteine pool (data from Table 1), it appears that the oxidation state of carbonic anhydrase III is similar to or slightly higher than the entire protein pool.

Discussion

The experiments presented here suggest that three distinct mechanisms for protection of protein cysteines may be important during oxidative stress (Figure 13). First, an oxidant can react directly with a protein molecule (probably by oxidation of a variety of different exposed protein structural components), resulting in the formation of a partially oxidized protein cysteine residue that is activated for further reaction with other cellular constituents. A protein thiol radical, protein cysteine thioperoxide, pro-

![Fig. 13](image)
tein cysteine thioperoxyradical, or protein cysteine sulfenic acid (Stortz et al., 1990; Thomas et al., 1995b; Denu and Tanner, 1998; Winterbourn and Metodiewa, 1999) might represent the partially oxidized protein cysteine. This modified cysteine most frequently reacts with GSH to form the S-glutathiolated protein because of the abundance of GSH as a reactant. This mechanism suggests an antioxidant role of GSH that has not previously been appreciated. Thus, if the concentration of GSH is decreased, the activated protein sulphydryl intermediate might instead react with molecular oxygen or some other oxidant which can result in further oxidation of the cysteine to forms that seem to be metabolically inert, i.e., irreversibly oxidized. Protein cysteine sulfenic or cysteine sulfonic acid (Figures 2 and 6) are possibly the major highly oxidized forms of cysteine present in normal cells. Second, the oxidant may react first with GSH to form an activated form of glutathione, which then reacts with protein cysteine to form S-glutathiolated protein. Finally, the oxidant might react with glutathione exclusively and thereby form significant amounts of GSSG. Since GSSG can potentially react with protein sulphydryls to form S-glutathiolated species, the possibility of protein modification by this mechanism is dependent on formation of significant amounts of GSSG. However, the reaction of GSSG with carbonic anhydrase III (and other proteins as well) appears to be too slow to generate significant S-thiolated protein in cells (Mallis et al., 2001). Although these mechanisms are all of potential importance, most data suggest that the first mechanism is of primary importance in vivo. Since this mechanism also provides a unique hypothesis for the potential role of glutathione as a trapping antioxidant for damaged protein sulphydryls, it is well suited to explain the results of the experiments reported in this manuscript.

Oxidation of carbonic anhydrase III with HOCl, AAPH or H2O2 produced considerable irreversible oxidation in the absence of GSH. Multiple acidic bands are generated on the IEF separation which are not reducible by DTT treatment. These bands also occur in oxidation of carbonic anhydrase III by a variety of other mechanisms (Lii et al., 1994; Thomas et al., 1995a; Thomas and Mallis, 2001). Reactions between thiols and reactive oxygen species often result in the formation of sulfinic or sulfonic acid (Wefers and Sies, 1983; Miller and Claiborne, 1991; Yeh et al., 1996; Becker et al., 1999; Hamann, et al., 2001), both of which are negatively charged sulfur compounds. Indeed, HOCl, AAPH and H2O2 treatment of carbonic anhydrase III all result in the formation of protein cysteine sulfenic acid (Figure 2) or both sulfinic and sulfonic acid (Figures 6 and 8). GSH prevented irreversible oxidation by AAPH or H2O2 and simultaneously participated in S-glutathiolation of carbonic anhydrase III (Figures 2 and 6). The protection of carbonic anhydrase III and formation of S-glutathiolated protein was dependent on the concentration of GSH relative to the concentration of the protein. Additionally, as S-glutathiolation increased, irreversible oxidation decreased in a GSH-dependent manner. This suggests that S-glutathiolation is linked to the prevention of irreversible oxidation (Park and Thomas, 1988; Li et al., 1994; Thomas et al., 1995b). Indeed, when S-glutathiolated carbonic anhydrase III was treated with the oxidants used in this study, formation of irreversibly oxidized carbonic anhydrase III was not observed (Figures 1, 4, and 6). While it is not unreasonable to expect that a disulfide bond between protein cysteine and glutathione could be oxidized to forms such as thio-sulfinate esters, it is probable that these reactions are significantly less favorable than oxidation of a protein thiol to a disulfide.

At lower GSH concentrations, initial oxidation of both proteins and glutathione are possible, since S-glutathiolated protein is a measured endpoint in both reactions. The increase in total protein modification at low GSH concentrations suggests either that initial formation of oxidized glutathione is an important mechanism, or that formation of irreversible oxidation products is less favored than the S-glutathiolation reaction. Because formation of cysteine sulfenic acid and cysteine sulfonic acid require multiple oxidation events, this supposition is reasonable. S-glutathiolation of carbonic anhydrase III remained extensive when GSH was several-fold more concentrated than the protein. This indicates that interaction of oxidant with the protein is important. The fact that irreversible oxidation occurs and that levels of irreversible oxidation in the absence of GSH are comparable to levels of total oxidation in the presence of GSH is further evidence that the protein reacts directly with the oxidant.

GSSG formation is not an important mechanism of S-glutathiolation in HOCl, AAPH or H2O2-mediated oxidation, and so GSSG formation may be important only at very high GSH:protein ratios. This corroborates studies with purified H-Ras in reactions with H2O2 and GSH (Mallis et al., 2001), suggesting that this is a general phenomenon for proteins and not limited to carbonic anhydrase III. In reactions using rat liver carbonic anhydrase III (Thomas and Mallis, 2001), identical molar ratios of GSH:protein (approximately 10:1) produced a wide variety of results depending on the oxidant. This shows that carbonic anhydrase III is an efficient scavenger of reactive oxygen species, but that its scavenging ability is heavily dependent on the nature of the oxidant.

The reaction of H2O2 with carbonic anhydrase III and cysteine shows that cysteine is as effective as an S-thiolating agent as GSH when carbonic anhydrase III is the protein substrate. If carbonic anhydrase III is typical of cytosolic proteins with reactive cysteines, then the predominance of S-glutathiolated proteins in oxidized cells is likely the result of the greater concentration of GSH in cells (Chai et al., 1994). Since carbonic anhydrase III does not appear to have a binding site for GSH (Mallis et al., 2000), this result should be relevant to many surface-exposed cysteine sites on proteins.

A previous study using BSO to deplete glutathione in hepatocytes had shown formation of irreversibly oxidized carbonic anhydrase III with menadione (Li et al., 1994).
The present experiments suggest that a minimal depletion of cellular glutathione may allow menadione to cause irreversible oxidation of carbonic anhydrase III. Further depletion results in extensive irreversible oxidation. Using DEM to deplete cellular glutathione has the advantage over using BSO in that the depletion occurs within 20 minutes. Thus, the irreversible oxidation does not seem to be a result of long-term secondary effects of glutathione depletion, but rather is likely to be the direct result of GSH deficiency. The results obtained with the purified protein show that decreasing the amount of available GSH is sufficient to result in irreversibly oxidized protein instead of S-glutathiolated protein and thus, the available GSH is sufficient to result in irreversibly oxidized protein. The results obtained with the purified protein show that decreasing the amount of available GSH is sufficient to result in irreversibly oxidized protein instead of S-glutathiolated protein and thus, the same is likely to be true in vivo. Because irreversibly oxidized carbonic anhydrase III increased slightly with menadione treatment after a minimal depletion of GSH, the concentration of GSH in hepatocytes may be just sufficient to protect the proteins in these cells from oxidative damage. This implies that in disease states where glutathione is depressed, there may be a significant impact on the protein pool even in situations when the depression is minimal. The persistence of irreversibly oxidized forms for over two hours after menadione challenge suggests that turnover of irreversible oxidation products is quite slow and buildup of damaged proteins can occur over time. This is borne out by the observed increase in irreversible modification of carbonic anhydrase III and in cysteine sulfenic acid content overall in rat livers of older animals versus younger animals. The fact that a variety of oxidants produced either cysteine sulfenic or sulfonic acid in vitro and that sulfenic acid is found in the proteins of young and old animals suggests that these irreversible modifications are important cellular modifications warranting further study.

Finally, the reaction mechanisms postulated here strongly suggest that regulation of proteins can occur through S-glutathiolation without the necessity of forming GSSG as an intermediate. This makes S-glutathiolation a plausible initial event in the activation of signaling cascades by oxidants. While the prevention of irreversible oxidation by S-glutathiolation has obvious value in preserving the function of cysteine-containing proteins, progressive irreversible oxidation of cysteines may contribute to degeneration of signaling seen in aging, cancer and other disease states (Liu et al., 1996; Rattan and Clark, 1996; Barrett et al., 1999a, b; Navarro et al., 1999).

**Materials and Methods**

**Materials**

L-cysteine, dithiothreitol (DTT), reduced glutathione (GSH), glutathione disulfide (GSSG), and N-ethylmaleimide (NEM), galactose, dexamethasone, collagen (type VII, from rat tail), bovine serum albumin, diethyl maleate, buthionine sulfoximine (BSO), sodium selenite, 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), NADPH, glutathione reductase (type III, from baker’s yeast), iodoacetamide, iodoacetic acid (IAA), N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), phenylmethylsulfonyl fluoride (PMSF), and leupeptin were from Sigma Chemical Company (St. Louis, USA), 2,2’-Azobis(2-amidinopropane)dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, USA). Sulfosalicilic acid dihydrate and sodium hypochlorite was from Aldrich Chemical Company, Inc. (Milwaukee, USA). Leibovitz’s L-15 medium with L-glutamine, bovine insulin, human transferrin, penicillin G, and streptomycin sulfate were from Gibco-BRL (Grand Island, USA). Collagenase (type I) was obtained from Worthington Biochemical Corp. (Freehold, USA). Amphotoles (pH 5–8 and pH 4–6) and Percol were obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, USA). PVDF membrane was from Millipore Corporation (Bedford, USA), Net-Filter for PAG was from Serva Biochem, Inc. (Westbury, USA). O-2-(maleimidoethyl)-O’-methylpolyethylene glycol 5000 (MalPEG) was obtained from Shearwater Corp. (Huntsville, USA). Purified recombinant human erythrocyte carbonic anhydrase III was the generous gift of D.L. Silverman (University of Florida, Gainesville, USA).

**Protein Assay**

Protein concentrations were determined as described by Lowry et al. (1951).

**Isoelectric Focusing (IEF) of Carbonic Anhydrase III**

Purified carbonic anhydrase III was separated on horizontal slab gels [5.0% (acylamide)/2.7% bisacylamide)/0.3% ampholyte pH 4.0 – 6.0/1.7% ampholyte pH 5.0 – 8.0] at 1500 V and 1.1 W/cm for 50 minutes as previously described for rat liver carbonic anhydrase III (Chai et al., 1991). The reduced form of the human erythrocyte enzyme separated at pI 7.6, a slightly more basic pI than the rat enzyme (reduced form pI=7.0) used previously in this laboratory (Chai et al., 1991). Gels were stained with Coomassie Brilliant Blue R-250 and air-dried.

**Quantification of IEF Gels**

Gels were scanned and bands were quantified using ImageQuant v3.3 (Molecular Dynamics Inc.). The extent of modification of carbonic anhydrase III reactive cysteines was calculated from the following relationship:

\[
\text{% modification} = \frac{(\text{density of band with 1 oxidized cysteine} + 2 \times \text{density of bands with 2 oxidized cysteines})}{(2 \times \text{density of all bands})} \times 100%
\]

Reversible modification is the difference in % modification between DTT-untreated and DTT-treated lanes. Irreversible modification is the % modification in DTT-treated lanes.

**Protein Sulfinic and Sulfonic Acid Analysis**

Samples were analyzed for irreversibly oxidized forms of cysteine (Hamann et al., 2001). Briefly, samples were denatured by incubation at 37 °C for 15 min in 120 μM phosphate buffer, pH 7.4 containing 7 M urea and 10 mM DTT. The samples were then treated with 40 mM IAM and incubated at room temperature for 20 min to block any remaining reduced cysteine residues. Each sample was split into two fractions, one of which was treated with 10 mM HOCl for 5 min at room temperature in order to oxidize sulfenic acids to sulfonic acids. The reactions were terminated after five minutes with the addition of 30 mM DTT. The samples were extensively dialyzed and hydrolyzed by vapor phase acid. Amino acid analysis was conducted using precolumn derivatization with OPA, and the amino acids were separat-
ed by reverse phase HPLC. Individual samples of hydrolyzed carbonic anhydrase III were compared by determining the moles of leucine in each sample. Since carbonic anhydrase III contains 22 leucine residues, the total leucine was divided by 22 to determine the moles of carbonic anhydrase III. The percent of reactive cysteine converted to cysteic acid was calculated according to the following relationship:

\[
\% \text{ cysteic acid} = \left( \frac{\text{mole of cysteic acid}}{\text{2 reactive cysteine residues}} \times \frac{\text{mol of carbonic anhydrase III}}{100} \right) \times 100\%
\]

HOCl converts sulfenic to sulfonic acid; therefore, the percent of cysteic acid found in a sample treated with HOCl represents the percent of total irreversibly oxidized reactive cysteine. Cysteic acid found in samples not treated with HOCl represents the percent of reactive cysteine detected as protein sulfonic acid. The percent of protein sulfenic acid can be calculated by subtracting the total protein sulfonic acid from the total irreversibly oxidized cysteine.

Liver protein extracts were compared on the basis of total cysteine content. The molar amount of cysteic acid detected was divided by the total mol of cysteine detected.

### Hepatocyte Isolation and Culture

Male Sprague-Dawley rats (200–250 grams) were from Sasco Co. (Omaha, USA). Water and a crude cereal-based diet were offered freely. Hepatocytes were isolated by perfusion with 0.05% collagenase as described (Thomas et al., 1995a). A male rat was anesthetized with secobarbital sodium (100 mg/kg body weight), and the liver was perfused at 37°C through the portal vein with 0.05% Type I collagenase. After approximately 15 min of perfusion, the liver was removed, sliced through nylon mesh, and cells were washed with L-15 medium. The cells were suspended in a 10% Percoll solution and centrifuged to separate parenchymal cells from dead cells. Isolated hepatocytes (0.5–10^6 cells/ml) were plated on collagen precoated culture plates in medium A (Leibovitz’s L-15 medium, pH 7.6, supplemented with 18 mM HEPES, 0.2% bovine serum albumin, 5 µg/ml insulin/transferrin, 1 µM dexamethasone, 5 mg/ml galactose, 5 ng/ml sodium selenite, 100 IU/ml penicillin G, and 100 mg/ml streptomycin sulfate). Cell viability was greater than 90% by Trypan blue exclusion. The culture medium was changed after 5 h to remove dead and unattached cells and again after 24 h. Hepatocytes were cultured for 48 h before starting experiments.

Hepatocytes that would be treated with diethyl maleate (DEM) and menadione were first incubated in medium B (Leibovitz’ Medium without BSA, insulin, transferrin and dexamethasone) for 1 h. DEM was added to this culture medium and the medium was replaced with fresh medium B for subsequent cell treatment. After a 15 min equilibration, menadione (in a DMSO vehicle) was added to a final concentration of 0.2 µM. The final concentration of DMSO was 0.2% in the medium. Since DMSO did not cause protein S-thiolation, alter cellular glutathione, or alter the cellular response to menadione, it was not directly added to control cultures in the experiments described here. Menadione was left on the cultures for 15 min, then the medium was removed, and cells were incubated in medium A for up to 24 h.

### Hepatocyte Extract Preparation

If glutathione was to be determined, experiments were terminated by rinsing the cells twice with cold phosphate-buffered saline (PBS). Cells were immediately placed in 250 µl cold buffer containing 20 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, and 5 µg/ml leupeptin, pH 7.4. Cells were completely disrupted with a small cell homogenizer, and homogenates were centrifuged at 10,000 g at 4°C for 30 min. An aliquot of each culture extract was treated with sulfosalicylic acid (final concentration was 10%) and the acid-soluble material was used to measure total glutathione by the DTNB-glutathione reductase recycling method (Tietze, 1969).

For preparation of protein extracts of hepatocytes, washed cell cultures were lysed as above in 20 mM HEPES, 5 mM EDTA, and 5 mM EGTA, pH 7.4, containing 50 µM N-ethyl maleimide. N-ethyl maleimide reacts with protein and non-protein sulphydryls rapidly to prevent artifactual modification of protein sulphydryls during sample preparation. Two aliquots of each sample were prepared, one of which was treated with 50 mM dithiothreitol at 37°C for 20 min before separation on electrofocusing gels.

### Electrofocusing/Western Blot Analysis of Protein Extracts

The molecular forms of carbonic anhydrase III in protein extracts were determined by separation on IEF gels as described above. This separation was combined with antibody detection after transfer to PVDF membranes as previously described (Lii et al., 1994; Thomas et al., 1995). The electrofocusing gel containing NetFix was separated from the Gelbond, equilibrated with cold 0.7% acetic acid for 15 min, and transferred at constant voltage, 12 V or 15 V (3 mA/cm²) for 30 min in a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Hercules, USA). Because proteins have a net positive charge in acetic acid, they were transferred to the membrane placed on the cathodic side of the gel. The PVDF membrane was washed with 150 mM NaCl, 15 mM Tris-HCl, pH 7.4, containing 0.3% Tween-20, and incubated with a rabbit anti-carbonic anhydrase III antiserum (supplied by S. Hendrich, Iowa State University, USA). Anti-rabbit IgG alkaline phosphatase conjugate from Sigma Chemical Company, along with p-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, were used to visualize the bound carbonic anhydrase III antibody.

### Lactate Dehydrogenase Activity

Medium was collected from hepatocytes and lactate dehydrogenase activity was measured directly with LDH lactate-dehydrogenase (EC 1.1.1.27) UV-test from Sigma. Total cellular lactate dehydrogenase was determined by lysing washed cells in 250 µl 20 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 1 mM benzamidine, and 5 µg/ml leupeptin, pH 7.4 buffer. The particle-free cellular supernatant was prepared by centrifugation and used to determine lactate dehydrogenase activity.

### Preparation of Soluble Liver Protein Extracts from Young and Old Rats

One month and 16 month old male, and one month and 23 month old female Fisher 344 rats were used. Food (Teklad Diet # 7002) and water were provided ad libitum. Rats were sacrificed by CO₂ inhalation, the liver was removed, rinsed with ice cold saline, and frozen immediately using a liquid nitrogen cooled clamp. Livers were powdered using a liquid nitrogen cooled stainless steel mortar and pestle and stored at –80°C until use. Soluble liver protein extracts were prepared by homogenizing liver tissue in ice-cold homogenization buffer (20 mM β-glycerophosphate, 10 mM DTT, 5 mM EDTA, and 5 mM EGTA, pH 7.4). The homogenate was centrifuged at 16 000 g for 10 min at 4°C, the supernatant was recovered, and centrifuged in a Beckman Airfuge Ultracentrifuge at 160 000 g for 30 min (twice). The soluble protein extract produced by this procedure was incubated at 37°C for 30 min to remove any DTT reversible modifications prior to protein sulfenic and sulfonic acid analysis or MalPEG analysis.
GainPEG Analysis of Protein Extracts
Soluble liver protein extracts were diluted ten-fold in 20 mM β-glycerophosphate buffer, pH 7.4, containing 5 mM GainPEG. Samples were applied to a 10% SDS-PAGE gel. Proteins were transferred to PVDF membrane, and carbonic anhydrase III was detected as described above, with the exception that the gel was equilibrated in buffer containing 48 mM Tris, 39 mM glycine, 1.3 mM SDS, and 20% methanol prior to transfer. Anti-rabbit IgG horseradish peroxidase conjugate with ECL chemiluminescent detection from Amersham Pharmacia Biotech (Piscataway, USA), was used to visualize the bound carbonic anhydrase III antibody.

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